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The Water-soluble Polysaccharide of Opium Poppy (*Papaver somniferum* L.). Identification of Acidic Oligosaccharides from a Partial Acid Hydrolysate

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The water-soluble polysaccharide extracted from opium poppy capsules was subjected to partial hydrolysis with 1 N sulphuric acid. The acidic oligosaccharides formed were separated on an anion exchange resin (De-Acidite) column in formate form, followed by filter-sheet chromatography. Of the six compounds isolated one (oligosaccharide I) is not known to have been described previously. The following sugars were obtained:

I. *O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-(1→2)-*O*-β-D-xylopyranosyl-(1→2)-D-xylose;
II. *O*-(α-D-galactopyranosyluronic acid)-(1→2)-L-rhamnose;
III. *O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-(1→4)-D-galactose;
IV. *O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-(1→2)-D-xylose;
V. *O*-(β-D-glucopyranosyluronic acid)-(1→4)-L-fucose;
VI. *O*-(α-D-galactopyranosyluronic acid)-(1→4)-D-galacturonic acid.

The capsule of the opium poppy, important because of its content of morphine and other alkaloids, is known to contain substantial amounts of a water-soluble complex polysaccharide.[1] Examination of aqueous extracts from fresh and dried capsules confirmed the complexity of the high-molecular carbohydrate material which, in its properties and composition, reminded much of a pectin.[2] Apart from the separation of a small proportion (0.4 %) of an almost pure galacturonan the aqueous extract of the opium poppy persistently resisted fractionation into different polysaccharide entities, thus indicating that the predominant part of the material constituted a heteropolysaccharide. This contained D-galactose, L-arabinose, D-xylose, L-rhamnose, D-galacturonic acid, 4-*O*-methyl-D-glucuronic acid, and traces of L-fucose, 2-*O*-methyl-L-fucose, 2-*O*-methyl-D-xylose and D-glucuronic acid.

Partial acid hydrolysis of the polysaccharide furnished a number of acidic oligosccharides, and the present paper reports the isolation and characterization of some of these compounds.

The general structure of a pectin-type polysaccharide implies the presence in the polymer of contiguous 1,4-linked α-D-galacturonic acid residues, and partial acid hydrolysis of pectins generally gives digalacturonic acid, trigalacturonic acid, and even higher homologues. The presence of this type of oligosaccharide in hydrolysates of the opium poppy capsular polysaccharide was demonstrated recently.[2] Since apparently 4-*O*-methylglucuronic acid has not been found previously in a pectin, it seemed of particular interest to obtain oligosaccharides containing this uronic acid.

Attempted enzymolysis of the polysaccharide with a commercial pectinase preparation proved unsuccessful, liberating minute amounts of galacturonic acid and only trace quantities of acidic oligosaccharides. Besides, the oligosaccharides most likely to be formed from a pectin by pectinase incubation would be the usual oligomers of galacturonic acid. Accordingly, conditions were chosen for partial degradation with acid, preferably yielding products different from the traditional type of pectic oligosaccharides.

Even under mild conditions of hydrolysis the yield of neutral oligosaccharides was very low; therefore the present investigation includes acidic sugars only.

The mixture of oligosaccharides obtained after partial hydrolysis was partly separated on a De-Acidite formate column by elution with a formic acid gradient (Fig. 1).

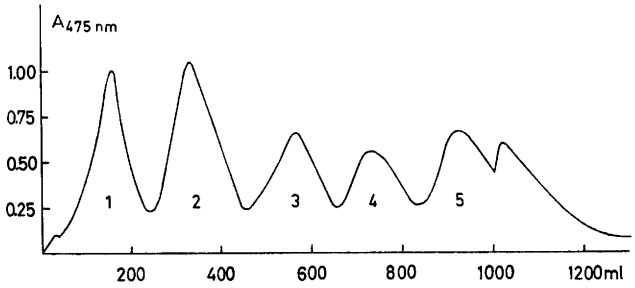

Fig. 1. Chromatography on De-Acidite FF-IP (SRA 67) in formate form of a partial acid hydrolysate of the opium poppy capsular polysaccharide. Gradient elution with formic acid as described in the text.

Compounds I, II, and III, together with galacturonic acid, appeared in the first peak of the elution curve, while the oligosaccharides IV, V, and VI were the major components of the second, third, and fifth peak, respectively. The fourth peak contained mainly 4-*O*-methyl-glucuronic acid. A complete fractionation on the column was not to be expected, considering the large number of acidic oligosaccharides present in the hydrolysate. However, refractionation of the sugars occurring in significant quantities by chromatography on thick filter paper resulted in the isolation of six oligosaccharides (compounds I to VI), of which two (II and III) were obtained in admixture.

I. *O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-(1→2)-*O*-β-D-xylopyranosyl-(1→2)-D-xylose;
II. *O*-(α-D-galactopyranosyluronic acid)-(1→2)-L-rhamnose;
III. *O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-(1→4)-D-galactose;
IV. *O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-(1→2)-D-xylose;
V. *O*-(β-D-glucopyranosyluronic acid)-(1→4)-L-fucose;
VI. *O*-(α-D-galactopyranosyluronic acid)-(1→4)-D-galacturonic acid.

The constitution of the oligosaccharides was elaborated routinely by 1. hydrolysis to the component sugars before and after treatment with sodium hydridoborate, and 2. reduction of the carboxyl group of the uronic acid moiety and methylation analysis of the resulting neutral oligosaccharide. The methylated monosaccharides liberated by acid hydrolysis were examined by paper-, thin-layer-, and gas-liquid chromatography. It should be stressed that the type of glycosidic linkage assigned to the oligosaccharides was based on optical rotation or comparison with the optical rotation of authentic compounds.

The aldotriouronic acid (I) has not been described previously as far as the authors are aware. Methylation of the derived neutral oligosaccharide methyl glycoside and subsequent acid hydrolysis gave 2,3,4,6-tetra-*O*-methylglucose and only one methylated xylose corresponding to 3,4-di-*O*-methylxylose, thus revealing that both of the xylose residues were linked through position 2. The lower rotation of the aldotriouronic acid ($[\alpha]_D + 5°$) as compared with that of the related aldobiouronic acid (oligosaccharide IV, $[\alpha]_D + 92°$) indicated that the xylobiose moiety in the former had a negative rotation and a β-glycosidic linkage. Oligosaccharide I had a chromatographic mobility and an optical rotation markedly different from that recorded for the aldotriouronic acid *O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-(1→2)-*O*-β-D-xylopyranosyl-(1→4)-D-xylose, which has been isolated from various hemicelluloses of the xylan type after partial acid hydrolysis [3,4] or enzymic degradation.[5]

Oligosaccharide IV was found to be identical with the aldobiouronic acid which frequently has been demonstrated to constitute a structural feature in several xylan hemicelluloses.[6,7] A 1,4-linked β-D-xylose polymer carrying 4-*O*-methyl-D-glucuronic acid residues at position 2 on some of the xylose units on graded hydrolysis would give aldobiouronic acid IV and, to a smaller extent, the aldotriouronic acid mentioned above. The finding of oligosaccharide IV as an integral part of a pectin-type polysaccharide was unexpected. Apparently this sugar must be regarded as a structural unit also of plant polysaccharides, not strictly associated with the hemicelluloses.

Oligosaccharides II and III which were eluted in the first peak from the De-Acidite column moved as a single compound on paper chromatography in all the solvent systems tried and on electrophoresis in acetate buffer. However, electrophoresis in borate buffer separated the fraction into two components. The more slow-moving compound gave rise to a stronger spot on the electropherogram than did the faster-moving component. Acid hydrolysis of the mixture of the two oligosaccharides gave considerably more galacturonic acid and rhamnose than 4-*O*-methylglucuronic acid and galactose; treatment with sodium hydridoborate followed by acid hydrolysis furnished rhamnitol

and galactitol in addition to the two uronic acids. From this oligosaccharides II and III evidently were aldobiouronic acids consisting of galacturonic acid and rhamnose and of 4-*O*-methylglucuronic acid and galactose, respectively, and II was the slower-moving compound on borate electrophoresis. Methylation analysis of the mixture after conversion to the neutral disaccharide methyl glycosides yielded 3,4-di-*O*-methylrhamnose, 2,3,6-tri-*O*-methylgalactose, 2,3,4,6-tetra-*O*-methylgalactose and 2,3,4,6-tetra-*O*-methylglucose. The mixture of the two aldobiouronic acids had $[\alpha]_D + 89°$. *O*-(α-D-Galactopyranosyluronic acid)-(1→2)-L-rhamnose, $[\alpha]_D + 84°$,[8] has been isolated from partial hydrolysates of a number of pectins, and *O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-(1→4)-D-galactose, $[\alpha]_D + 102°$,[9] has been found to be a component of partial hydrolysates from various plant gums. It was of interest to note that Aspinall *et al.*[10] obtained a mixture of the same two aldobiouronic acids (II and III) from a partial hydrolysate of *Khaya grandifolia* gum. Not either in that case the authors succeeded to separate the compounds by chromatographic methods, and the structural studies were carried out on the mixture of the two sugars.

The isolation of oligosaccharide V in a yield comparable to that of the other oligosaccharides was surprising, considering that glucuronic acid and fucose occurred as trace components in the polymer. The methylation experiment was unsuccessful, making it difficult to ascertain the mode of linkage in the disaccharide. However, the sugar gave a positive staining reaction with triphenyltetrazolium hydroxide, indicating a free hydroxyl group in the 2-position of the fucose residue.[11] The corresponding 1,3-linked aldobiouronic acid, *O*-(β-D-glucopyranosyluronic acid)-(1→3)-L-fucose, $[\alpha]_D - 18°$,[12] a major feature of the cell-wall polysaccharide of the brown alga *Ascophyllum nodosum*, has a different rotation and a different chromatographic and electrophoretic mobility from oligosaccharide V. On the other hand the opium poppy glucuronosylfucose had an optical rotation and a chromatographic and electrophoretic mobility corresponding to *O*-(β-D-glucopyranosyluronic acid)-(1→4)-L-fucose, recently isolated from partial hydrolysates of soy-bean meal polysaccharide[13] and lemon-peel pectin,[14] and accordingly oligosaccharide V was given tentatively that structure.

The digalacturonic acid (VI) was, together with the aldobiouronic acid (II), the only typical pectic oligosaccharide obtained. The optical rotation, chromatographic mobility and chemical characterization were in agreement with the proposed structure, *O*-(α-D-galactopyranosyluronic acid)-(1→4)-D-galacturonic acid.

The content in the polysaccharide of 4-*O*-methylglucuronic acid and galacturonic acid was 4 % and 60 %, respectively, and the preferential formation of oligosaccharides containing the former acid under the conditions used agrees with the earlier finding[2] that 4-*O*-methylglucuronic acid occupy end groups in the polymer. On the other hand the galacturonic acid residues presumably occur in the inner part of the molecule as a polygalacturonic acid backbone which would require more drastic conditions to be extensively degraded into low-molecular weight oligosaccharides. Arabinose was the only neutral sugar of the polysaccharide not present in any of the oligosaccharides isolated. The absence of arabinose-containing oligosaccharides in-

dicates, as suggested previously,[2] that a major part of this sugar occurs as acid-labile arabinofuranose residues.

It is evident from the studies carried out so far that the opium poppy capsular polysaccharide is a highly complex polymer, and more information is required to attain a clearer picture of its molecular structure.

EXPERIMENTAL

Paper chromatograms were run on Whatman No. 1 and, for preparative purposes, on Whatman No. 3 MM filter paper in the following solvent systems (v/v):

A. Ethyl acetate, acetic acid, formic acid, water, 18:3:1:4.
B. Ethyl acetate, pyridine, acetic acid, water, 5:5:1:3.
C. Butanol, ethanol, water, 40:11:19.

$R_{\text{Gal A}}$ is the rate of movement relative to galacturonic acid.

Thin layer chromatography (TLC) was carried out on Kieselgel G in benzene, acetone, 1:1 (v/v).

Gas-liquid chromatography (GLC) of methylated methyl glycosides [15] was performed on 120 × 0.5 cm columns in a Pye Argon chromatograph using 15 % w/w of butane-1,4-diol succinate polyester on acid-washed Celite (column 1) at 175° and 10 % w/w of polyphenyl-ether [*m*-bis-(*m*-phenoxyphenoxy)-benzene] on acid-washed Celite (column 2) at 175°. GLC of trimethylsilylated (TMS) sugars [19] was carried out on column 2 at 155° or on a 390 × 0.9 cm column in a Wilkens Aerograph Model 700 chromatograph using 3 % *w/w* of SE 30 on Chromosorb W (column 3) at 200°.

Zone electrophoresis was performed on Munktell No. 302 filter paper at *ca.* 40 V/cm in 0.05 M sodium tetraborate, pH 9.2, and in 0.10 M sodium acetate buffer, pH 6.4. $M_{\text{Gal A}}$ is the rate of migration relative to galacturonic acid.

Sugars were located on chromatograms and electropherograms with the following reagents:

a. Aniline oxalate, saturated aqueous solution.
b. Silver nitrate-sodium hydroxide.[16]
c. A freshly prepared solution of 2,3,5-triphenyltetrazolium chloride, 1 %, in 1 N sodium hydroxide.

Optical rotations were measured at 20° in water.

Small scale acid hydrolysis of oligosaccharides was carried out in 2 N sulphuric acid at 100° for 3—4 h (neutral sugars) or 8—10 h (acidic sugars).

Partial acid hydrolysis and isolation of oligosaccharides

The opium poppy was grown in the Botanical Garden of the University of Oslo; extraction and isolation of the polysaccharide material was carried out as described previously.[2] The polysaccharide (20 g) was hydrolysed with 1 N sulphuric acid (2 l) at 100° for 3 h. After neutralization of the hydrolysate with the calculated amount of barium hydroxide and treatment of the filtrate with Dowex-50 (H^+) the resulting solution was concentrated *in vacuo* and applied to the top of a column (4.5 × 62 cm) of De-Acidite FF-I P (SRA 67) in formate form. Neutral sugars were washed out with water, and elution of the column with a 0—2 N formic acid gradient (800 ml of each) gave fractions containing galacturonic acid, 4-*O*-methylglucuronic acid, and acidic oligosaccharides. Each fraction (5 ml) was analysed for carbohydrate content with the phenol-sulphuric acid method.[17] Further fractionation of the oligosaccharides was carried out by filter-sheet chromatography in solvents A or B.

Oligosaccharide I. The sugar (18 mg) had $[\alpha]_D + 5°$ (*c* 1.8), $R_{\text{Gal A}}$ 0.39 and 0.84 in solvents A and B and $M_{\text{Gal A}}$ 0.19 and 0.47 in borate buffer and acetate buffer, respectively. It gave no staining reaction with triphenyltetrazolium hydroxide. On acid hydrolysis oligosaccharide I gave xylose, 4-*O*-methylglucuronic acid, and oligosaccharide IV. Treatment of the sugar with sodium hydridoborate followed by acid hydrolysis

yielded xylitol, xylose, 4-*O*-methylglucuronic acid, and oligosaccharide IV. The sugar (10 mg) was converted into the methyl ester methyl glycoside by refluxing it with methanol and Dowex-50 (H^+) resin for 24 h. The product was reduced with lithium hydridoaluminate in tetrahydrofuran by gentle reflux for 2 h. Methylation [18] of the neutral oligosaccharide methyl glycoside with methyl iodide and barium oxide in dimethyl formamide gave a product (5 mg) which on acid hydrolysis and subsequent paper chromatography in solvent C and TLC had the mobilities of 2,3,4,6-tetra-*O*-methylglucose and 3,4-di-*O*-methylxylose; the glucose derivative and the xylose derivative were present in the approximate molar proportions 1:2. GLC on columns 1 and 2 of the methyl glycosides of the methyl sugars gave peaks with retention times corresponding to methyl 2,3,4,6-tetra-*O*-methylglucoside and methyl 3,4-di-*O*-methylxyloside, authentic compounds being run as references.

Oligosaccharides II and III. The chromatographically homogeneous fraction (25 mg) had $[\alpha]_D+89°$ (*c* 0.9); it had $R_{Gal\ A}$ 0.65 and 0.78 in solvents A and B, respectively, and it also moved as a single compound in the system butanol, acetic acid, water, 12:3:5 (v/v). It had $M_{Gal\ A}$ 0.65 in acetate buffer. On electrophoresis in borate buffer two spots were obtained (reagent a), having $M_{Gal\ A}$ 0.34 and 0.54, respectively. Acid hydrolysis of the fraction produced galactose, rhamnose, galacturonic acid, and 4-*O*-methylglucuronic acid. Reduction with sodium hydridoborate prior to acid hydrolysis gave galacturonic acid and 4-*O*-methylglucuronic acid and, in addition, two non-reducing compounds corresponding to galactitol and rhamnitol. The oligosaccharide fraction (15 mg) was converted into the methyl ester methyl glycoside, reduced with lithium hydridoaluminate and methylated. Paper chromatography of the acid hydrolysate in solvent C and TLC revealed spots (reagent a) with the mobilities and colours of 2,3,4,6-tetra-*O*-methylgalactose, 2,3,4,6-tetra-*O*-methylglucose, 2,3,6-tri-*O*-methylgalactose, and 3,4-di-*O*-methylrhamnose. The dimethylrhamnose gave a positive reaction with reagent c, while the trimethylgalactose gave no stain with this reagent. GLC on columns 1 and 2 of the methyl glycosides of the methyl sugars gave peaks with the retention times of the methyl glycosides of 2,3,4,6-tetra-*O*-methylgalactose, 2,3,4,6-tetra-*O*-methylglucose, 2,3,6-tri-*O*-methylgalactose, and 3,4-di-*O*-methylrhamnose, respectively, the authentic compounds being run as references.

Oligosaccharide IV. The sugar (72 mg) $[\alpha]_D+92°$ (*c* 2.1) had $R_{Gal\ A}$ 1.52 and 1.49 in solvents A and B and $M_{Gal\ A}$ 0.41 and 0.74 in borate and in acetate buffer, respectively. The oligosaccharide gave no staining reaction with reagent c; acid hydrolysis produced xylose and 4-*O*-methylglucuronic acid. Hydrolysis of the derived oligosaccharide alcohol gave xylitol and 4-*O*-methylglucuronic acid. The sugar (52 mg) was converted to the methyl ester methyl glycoside and reduced with lithium hydridoaluminate, yield 40 mg. Acid hydrolysis of a 10 mg portion of the oligosaccharide methyl glycoside furnished products indistinguishable from xylose and 4-*O*-methylglucose in their chromatographic and electrophoretic mobilities. Preparation of the TMS-derivatives [19] of the two sugars and subsequent GLC on column 3 gave peaks with the same retention times as the TMS-derivatives of the authentic compounds.

The remaining oligosaccharide methyl glycoside was methylated; a part of the product was methanolysed and subjected to GLC on columns 1 and 2, and the residual part was hydrolysed and examined by paper chromatography and TLC. In each case the results corresponded to the presence of 2,3,4,6-tetra-*O*-methylglucose and 3,4-di-*O*-methylxylose.

Oligosaccharide V. The sugar (21 mg), $[\alpha]_D-68°$ (*c* 1.0), had $R_{Gal\ A}$ 0.52 in solvent A, 0.78 in solvent B, $M_{Gal\ A}$ 0.58 in borate buffer and 0.49 in acetate buffer. The oligosaccharide which gave a positive reaction with reagent c, was hydrolysed by acid to glucuronic acid, glucuronolactone, and fucose. Hydrolysis of the derived oligosaccharide alcohol yielded products with the chromatographic mobility of fucitol, glucuronic acid, and its lactone. Conversion of the sugar into the neutral oligosaccharide methyl glycoside followed by hydrolysis gave glucose and fucose in approximate equimolar proportions. A 2 mg portion of the deionised hydrolysate was dissolved in 1 ml 0.1 M phosphate buffer pH 5.6 and incubated with 0.5 mg glucose oxidase (Sigma) at 35° for 15 h. Chromatographic examination of the digest revealed fucose as the only reducing sugar (reagent a) in addition to a compound with the mobility of gluconic acid.

Oligosaccharide VI. The sugar (28 mg) had $[\alpha]_D+123°$(*c* 1.0). It had $R_{Gal\ A}$ 0.19 and 0.21 in solvents A and B and gave galacturonic acid on hydrolysis. Conversion of the oligosaccharide into the methyl ester methyl glycoside followed by reduction with

lithium hydridoaluminate and acid hydrolysis gave galactose as the only sugar. Methylation of the neutral oligosaccharide methyl glycoside (16 mg) furnished a product, the half of which was methanolysed and the other half was hydrolysed. The two major compounds of the hydrolysate had the chromatographic mobilities of 2,3,4,6-tetra-*O*-methylgalactose and 2,3,6-tri-*O*-methylgalactose, respectively; the latter did not react with triphenyltetrazolium hydroxide. The methanolysis products, when subjected to GLC (columns 1 and 2) had the same retention times as the authentic methyl glycosides of 2,3,4,6-tetra-*O*-methyl-D-galactose and 2,3,6-tri-*O*-methyl-D-galactose.

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